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Report

Incidence of an estrogen receptor polymorphism in breast cancer patients

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Summary

We previously identified a polymorphism in the human estrogen receptor (ER) gene, within the coding region for the protein's amino terminal B-domain. In estrogen receptor-positive (ER^+) breast tumors, the variant allele was preferentially associated with lower levels of ER, and was clinically correlated with frequent spontaneous abortions. DNA sequencing revealed a point mutation that changes codon 86 from Ala to Val and a silent mutation in codon 87. Because we initially detected the variant allele by analyzing RNA, only those tissues in which the ER gene is actively expressed were suitable for genotype analysis. We now describe an assay that uses genomic DNA as the substrate for determining the ER B genotype; DNA containing the polymorphic region of the ER gene is amplified by the polymerase chain reaction, then the amplified DNA is hybridized with radiolabeled oligonucleotide probes complementary to the wild type and variant ER alleles. This method allowed us to determine the ER B genotype of women with ER^+ and ER^- tumors, starting with minute amounts of DNA from frozen or paraffin embedded tissues. ER B genotyping was also performed on women without breast cancer using DNA extracted from blood cells. The combined results from analyses of RNA and DNA from 300 breast cancer patients showed that 12% were heterozygotes. In the ER^+ group ($n = 183$), 11.5% carried the variant gene compared to 12.8% in the ER-negative group ($n = 117$) ($\chi^2 = 0.11$; $df = 1$; $p > 0.25$). No link to tumor histology could be established. Preliminary data on DNA from blood of healthy women over the age of 50 ($n = 64$) yielded a slightly lower ER B-variant frequency (9.4%); this frequency was not significantly different than that in the breast cancer groups. Thus, while the variant ER allele is associated with low ER levels in ER positive breast tumors, its frequency is not different in the ER^+ and ER^- tumor groups and may be unrelated to breast cancer development.

Introduction

A naturally occurring polymorphism in the human estrogen receptor gene was first identified by a RNA/RNA hybridization-nuclease protection assay of estrogen receptor (ER) mRNA in estrogen receptor positive (ER^+) breast tumors and non-tumorous hysterectomy samples [1]. The variant

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gene contains two nearby point mutations within the protein coding B-region of the receptor [2]. Subsequent analyses of women in the ER^+ tumor group revealed that those with the variant gene had a significantly increased incidence of spontaneous abortion [3]. This suggests that the variant ER gene product is associated with altered ER function.

Tumor ER status often serves as a criterion for selecting the mode of post-surgical treatment of breast cancer patients [4]. Also, tumor ER negativity has been associated with lower survival rates among breast cancer patients [4]. In this context, we noted that in the ER^+ tumor group, presence of the B-variant allele was correlated with low ER protein levels [1]. We therefore questioned whether the frequency of the variant gene might be greater in the group of women with ER^- tumors.

Most frozen breast tumor samples that lacked measurable amounts of ER protein also contained negligible amounts of ER mRNA [1, 5, 6]. This precludes using the assay of RNA for identifying ER-B variants. Therefore, in the current study we report an assay for detecting the ER B-variant genotype that uses genomic DNA rather than RNA as the diagnostic substrate. Specifically, a small segment of DNA around the region of the B-variant is amplified by the polymerase chain reaction (PCR) [7] and the ER B genotype is determined by allele specific oligonucleotide hybridization (ASO) [8].

This assay enabled us to identify ER B-variant women by analyzing DNA from ER^- breast tumors. Moreover, the ASO assay of amplified DNA allowed us to determine the ER B genotype of any individual from whom we could obtain a DNA sample. This included individuals whose breast tumors had been preserved in paraffin blocks and individuals who, irrespective of whether they had breast cancer, volunteered to provide blood samples.

Methods

Subjects

All subjects in the current study were women. Breast tumor samples, both frozen and paraffin embedded specimens, were obtained through the Department of Pathology at Mt. Sinai Hospital. Blood samples from women who did not have breast cancer were obtained in the Department of Obstetrics and Gynecology at Mt. Sinai Hospital and at Long Island Jewish Hospital; these samples were obtained from both clinic and private patients after receiving their informed consent.

DNA extraction

One hundred twelve DNA samples were extracted from sections of formaldehyde fixed, paraffin embedded breast tumors that were up to three years old. DNA was isolated using the method of Wright and Manos [10]. In general, three tissue sections were digested in 100 μl of digestion buffer.

In 13 cases, DNA was isolated from the same guanidine isothiocyanate homogenates of frozen tumors previously used to obtain RNA [11]. Here, after ultracentrifugation over a CsCl cushion, the DNA at the interface was recovered and stored at $-20°$ C until needed, then dialysed against 10 mM Tris, 0.1 mM EDTA, pH 8.0 (TE), and further purified by treatment with 50 μg/ml protease K in 100 mM Tris, pH 8.0, 50 mM EDTA, 0.5% SDS at 37° C for 4 hr, extracted with phenol/$CHCl_3$, then $CHCl_3$ alone, then precipitated with ammonium acetate and ethanol. DNA was redissolved in a small volume of TE.

All other DNA samples were prepared from blood cells. Five ml blood were collected into EDTA-containing tubes, and tubes were stored at 4° C for 5 hr to 3 d in an upright position. Serum was discarded and the 'buffy coat' interface, along with a small amount of overlying serum and underlying red blood cells, was transferred to a 5 ml capped tube. Tubes were filled with cell lysis buffer (10 mM Tris, pH 7.5, 640 mM sucrose, 5 mM $MgCl_2$, 1% Triton X-100), mixed, then centrifuged for 20 min at 8000 × g, 4° C. Nuclear pellets were recovered and resuspended in 0.45 ml 75 mM NaCl, 25 mM EDTA, pH 8.0, and processed essentially as described above.

DNA isolation was carried out in separate facilities and with separate equipment from those used

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DNA isolation was carried out in separate facilities and with separate equipment from those used

for analysis of amplified products. This minimized the likelihood of contaminating unamplified stocks with amplified DNA products.

DNA amplification

A 151 BP fragment of genomic DNA containing the polymorphic portion of the B region of the estrogen receptor gene was amplified by the polymerase chain reaction (PCR) using previously described oligonucleotide primers [2]. Cetus-Perkin Elmer Taq polymerase, buffer, and deoxynucleotides were used as recommended by the supplier. Reaction conditions were 1 min each at 95° C, 60° C, and 72° C for 30 cycles with a final extension cycle at 72° C for 10 min. Input template was 1–2 μg of DNA purified from blood or frozen tissue and 10 μl of extract from paraffin sections. In initial experiments each DNA was amplified in a 100 μl reaction volume; later studies used 50 μl per sample, which was more than sufficient for rapid analysis by Southern blotting of replicate samples.

Southern blotting and oligonucleotide hybridization

Replicate aliquots of amplified DNA were fractionated on 2% agarose gels (IBI; Molecular Biology Grade) in Tris/borate/EDTA buffer [12]. DNA was visualized by ethidium bromide staining, processed for transfer to membranes (Nytran or Gene Screen) using a standard protocol [12], and fixed by UV irradiation, or baking for 2 h at 80° C.

For hybridization, synthetic oligonucleotide probes corresponding to the wild type (tctgaggCggcGgcgttcggc) and the variant (tctgaggTggcCgcgttcggc) sequences [2] were used. Probes were radiolabeled with gamma ^{32}P ATP (~ 3000 Ci/mmole; NEN) to a specific activity of $> 10^8$ cpm/μg using polynucleotide kinase (New England Biolabs).

Filters were incubated in 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS for 1 h at 37° C with constant shaking [12]. Probe (1–2 × 10^6 cpm/ml final concentration) was added to each hybridization bag in a small amount of the same buffer, and bags were incubated overnight as above. Filters were washed three times with 6 × SSC at room temperature, once with 3 M tetramethyl ammonium chloride (TEMAC) at room temperature, then twice at 62–63° C in prewarmed 3 M TEMAC for 20 min [13].

RNase protection assay

RNA isolated from frozen breast tumor samples [11] was assayed for the presence of the ER B-variant using a solution hybridization assay essentially as previously described [1]. For this assay a subclone of the wild type ER cDNA covering the B-polymorphic region [1, 14] was used to synthesize ^{32}P-labeled antisense RNA. For hybridization, each tissue RNA sample (5–15 μg) was mixed with 200,000 cpm of probe (specific activity $> 10^9$ cpm/μg), hybridized, treated with RNase, and analysed on acrylamide gels as described [1]. With this probe, samples that were homozygous for the wild type ER B allele contained a nuclease resistant band on autoradiograms that is 260 BP; samples that were B-variant heterozygotes contained the 260 BP band, and in addition, had bands 150 and 110 BP. (See ref. 1, Fig. 5.)

Estrogen receptor assay

The ER status of each breast tumor was obtained from the laboratory of Dr. Swan Thung, Department of Pathology, Mt. Sinai School of Medicine. Samples are routinely assayed for ER using the dextran coated charcoal method (RIANEN Assay System). Tumors were scored as ER$^+$ if they contained > 2 fmol ER/mg cytosolic protein.

Results

Fifteen DNA samples from women whose tumors had been previously genotyped by solution hybridization/RNase protection [1] were tested in the PCR amplification/ASO hybridization assay using blood, or frozen or paraffin embedded tissue as a starting material. All samples previously scored as homozygous wild type (n = 10) in the RNA assay showed that same genotype in the DNA assay. All samples previously scored as ER B-variant heterozygotes (n = 5) by RNase protection proved to be heterozygotes in the DNA assay as well (data not

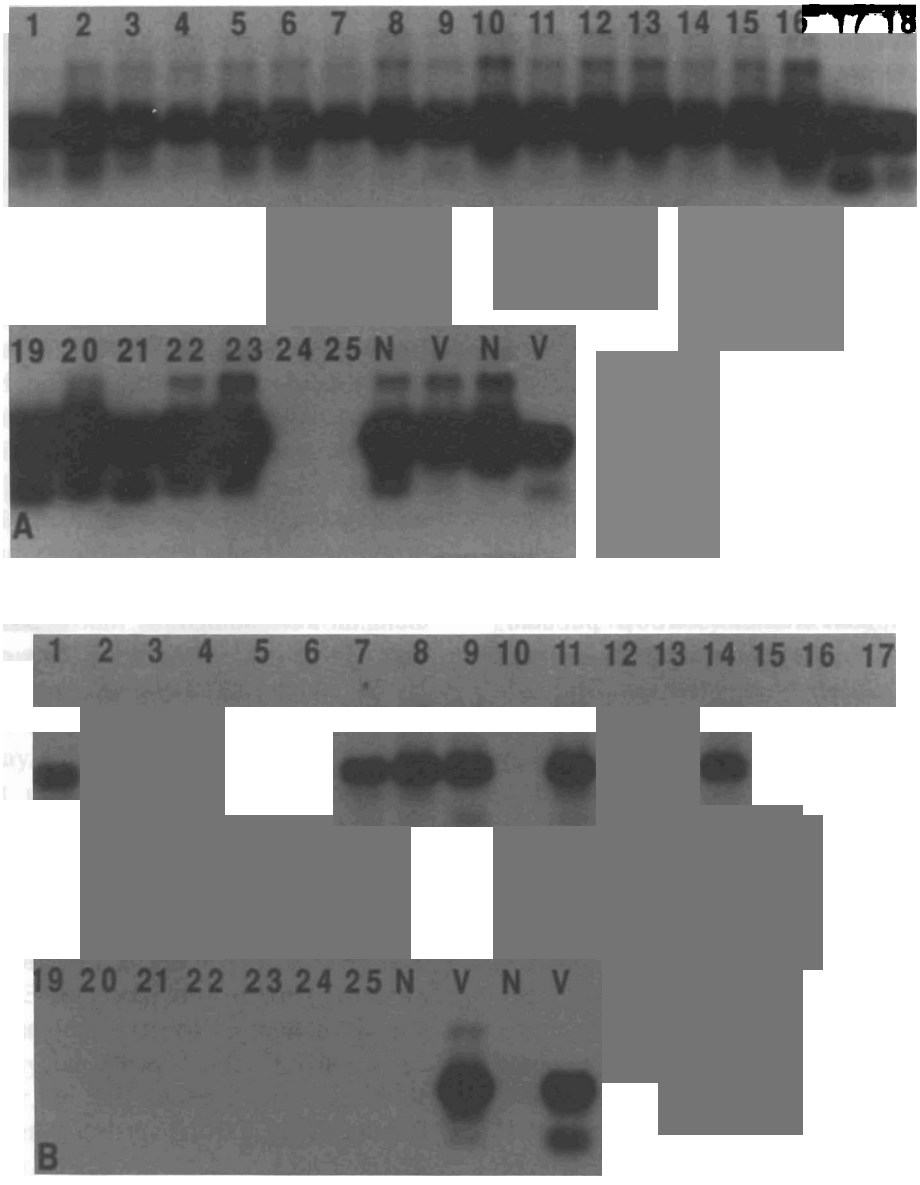

Fig. 1. Detection of the ER B-variant genotype in PCR amplified genomic DNA. The autoradiogram shows the results of replicate Southern blots. After 30 cycles of PCR, two Southern blots, each with 1/3 of the reaction mixture, were performed. The replicate filters were hybridized with the wild type oligonucleotide probe (Fig. 1A) or the variant probe (Fig. 1B). Samples homozygous for the wild type allele only give a signal in the hybridization with the wild type probe. Heterozygous samples, which carry one copy of each allele, gave a signal with both probes. Samples homozygous for the variant allele would hybridize only with the variant probe; none are shown on this gel. Lanes 1–13 – DNA isolated from blood cells; Lanes 14–23 – DNA isolated from frozen tumors; Lane 24 – negative control (plasmid DNA); Lane 25 – negative control (no template); Lanes N – positive wild type controls (DNA from known wild type homozygotes); Lanes V – variant positive controls (DNA from known variant heterozygotes). Positive controls were DNA samples from individuals whose genotype had been previously determined by RNase protection analysis of breast tumor RNA [1]. The homozygous wild type (N) controls bound only to the wild type oligonucleotide probe while the heterozygous variant (V) controls bound to both the wild type and variant probes. Samples in Lanes 1, 7, 8, 9, 11, 14 hybridized to both probes and were therefore classified as ER B-variant heterozygotes; all other test DNA samples were scored as wild type homozygotes.

shown). Therefore we concluded that genotype data obtained by the two methods could be combined to determine the frequency of the ER B-variant allele in the various study groups.

We then analyzed DNA from 108 more breast cancer patients by PCR/ASO only. The RNase protection assay was not possible on these patients, either because of a lack of ER mRNA in ER-negative tumors or because no frozen timor tissue was available. The results of a typical analysis are shown in Fig. 1. Included in this sample set are two DNA samples from individuals previously scored as homozygous wild type (labeled N) and two samples from individuals who were known heterozygous B-varients (labeled V). As seen in Fig. 1A, the wild type oligonucleotide probe gave signals with both the N and V samples. In contrast, as seen in Fig. 1B, the variant oligonucleotide gave signals with both V samples but not with either N sample. Neither probe gave signals in tests against PCR amplification reactions containing a heterologous plasmid DNA (Fig. 1A and B; Lane 24) or no added DNA template (Fig. 1A and B; Lane 25).

Table 1.

Tumor receptor status	ER^+		ER^-	
ER B-genotype	WT	Var	WT	Var
Method of genotype analysis				
RNAse only	130	11	30	3
RNAse & PCR/ASO	4	6	8	0
PCR/ASO only	28	4	64	12
Total	162	21	102	15
Frequency of ER B-variant allele		11.5%		12.8%

A total of 183 women with ER^+ breast cancer ($ER > 2$ fm/mg protein), and 117 women with ER^- breast cancer were screened for the presence of the ER B-variant allele. Women were genotyped either by the RNase protection assay of breast tumor RNA, by allele specific oligonucleotide hybridization of amplified genomic DNA (PCR/ASO), or by both methods, as indicated above. In all cases where an individual was genotyped by both methods, the results were internally consistent. Therefore data obtained by the two methods was pooled to compare the frequency of the ER B-variant genotype in the ER^+ and ER^- breast cancer groups; no significant difference was seen between groups ($X^2 = 0.165$, $df = 1$, $p > 0.25$).

These negative control samples were included to confirm that reagents for amplification were free of contaminating amplification products from previous experiments. Drawing on these results, the test samples could be assigned an ER B-genotype. Accordingly, in the assay shown in Fig. 1, samples in Lanes 1, 7, 8, 9, 11, 14 were scored as ER B-variant heterozygotes and all others were scored as ER B wild type homozygotes.

To determine the frequency of the ER B-variant genotype in the ER^+ and the ER^- breast tumor groups, data from the RNase protection assay [1] and data from the PCR/ASO were combined; samples genotyped by RNase protection included 66 samples described in ref. 1 and an additional 126 samples analysed by the same method (data not shown). The combined results of the RNase protection and the PCR/ASO studies showed that, in the ER^+ tumor group, 21 of 183 women (11.5%) carried the B-variant gene, and, in the ER^- breast tumor group, 15 of 117 (12.8%) carried the variant gene. (See Table 1, which gives genotype information on women in the ER^+ and ER^- tumor groups and documents the method(s) of analysis used to obtain these data.) These results show that there is no significant difference between groups in the frequency of the ER B-variant ($\chi^2 = 0.11$, $df = 1$, $p > 0.25$).

As shown in Table 1, 41 samples that were scored as ER^- by steroid binding, (38 homozygous wild type and 3 ER B-variant heterozygotes) contained sufficient ER mRNA to be readily genotyped by the RNase protection assay; this finding may reflect a greater sensitivity of the RNase protection assay, different amounts of ER gene expression in the tissue fragments used for the RNA and the protein assay, or mutations in the ER gene outside the region of the RNA probe used for the assay.

Inspection of patient pathology records revealed no correlation between the ER B-variant genotype and tumor staging or grading in either the ER^+ or ER^- tumor groups. One sample, from a primary tumor that was ER negative by both steroid binding and RNase protection, repeatedly scored as a B-variant homozygote in the PCR/ASO. However, subsequent analysis of lymph node DNA extracted from a paraffin block from this individual

proved to be heterozygous for the ER B-polymorphism, suggesting that a loss of heterozygosity had occurred in the tumor.

In a subpopulation of 64 healthy women over the age of 50 with no history of breast cancer and normal gynecological histories, six ER B-variant heterozygotes (9.4%) were detected by PCR/ASO analysis of DNA from blood; this frequency is slightly but not significantly lower than the ER B-variant frequency in the breast cancer group. (See Table 2.) Pooling data from this study as well as an ongoing investigation of other women currently gives genotype information on 483 women. Fifty-nine women in this entire group (12.2%) are ER B-variants, and all of them are heterozygotes. In both the breast cancer group and the nonbreast cancer group the ER B-variant allele was found in Caucasian (Hispanic and Nonhispanic) and Black women. Since the study has thus far included few women from other racial-ethnic groups and since we have not yet obtained the racial/ethnic data on all women who have been genotyped, no further information can be given on the ER B-variant frequency of these subgroups.

Discussion

Selective oligonucleotide hydridization of amplified DNA has been used to identify individuals carrying a variant form of the human estrogen receptor gene. This method of genotyping made use of DNA from unfixed, frozen and formalin-fixed, paraffin embedded breast tumors, as well as from blood lymphocytes. Hence, this method has enabled us to greatly expand the population of individuals whose ER B genotype can be readily determined.

As an initial application of this method, we compared the frequency of the ER B-variant gene in women with ER^+ and ER^- primary breast tumors. A motivation for this study came from the observation that, in the ER^+ tumor group initially studied, individuals with the variant gene had ER protein levels (measured by steroid binding) that were, on average, lower than those in the homozygous wild type group [1]. This suggested that the frequency of the variant gene might be greater among women with ER^- breast tumors. It also suggested that B-variant homozygosity might be more prevalent in the ER^- tumor group.

The results presented in this paper demonstrate that the frequency of the variant allele in the group of women with receptor negative tumors (12.8%) is not significantly higher than its frequency in the group with receptor-positive tumors (11.5%). Because the B-variant allele was found in approximately that same frequency (14.3%) in women in an unselected population, it appears that the variant allele is not a single dominant gene, associated with the development of breast cancer. We observed that the variant allele was slightly less prevalent (9.4%) in women over 50 years of age who have no history of breast or other gynecological cancers and who are in general good health. Further studies on larger sample groups are needed to determine whether the observed lower frequency of the ER B-variant allele in the group of cancer-free women is significant.

Given that the frequency of the ER B-variant gene is not significantly higher in the ER^- breast cancer group than in the group with ER^+ breast

Table 2.

ER B-genotype	WT	Var	Total
Group			
ER^+ cancer	162	21 (11.5)[a]	183
ER^- cancer	102	15 (12.8)	117
Healthy women > 50	58	6 (9.4)	53
All other women	102	17 (14.3)	119
Total	424	59 (12.2)	483

[a] Numbers in parentheses are percentages of group totals.

The incidence of the ER B-variant allele was compared among four groups of women: ER^+ breast cancer patients; ER^- breast cancer patients; women over the age of 50 without breast cancer or other gynecological cancers; and 'All other women'. The group of 'all other women' includes women under the age of 50 as well as women who had hysterectomies and therefore provided hysterectomy samples to use for comparing genotype analysis by RNase protection and PCR/ASO. Comparison between each pair of groups demonstrates that the ER B-variant allele is not significantly associated with breast cancer ($\chi^2 = 1.09$; $df = 3$; $p = 0.78$) and appears to be present in ~ 12% of the female population.

cancer, how might we explain the previous observation of a lower mean of ER protein in the ER^+ variant samples than in the ER^+ wild type breast tumor samples [1]? One possibility is that the variant allele does not encode a protein because it contains an additional, as yet unidentified, mutation in the coding sequence. Therefore, complete sequence analysis of the protein-coding region of ER B-variant mRNAs is needed.

A second possible reason for the low ER protein levels in ER^+ B-variant tumors could be that the variant ER protein is a weaker (direct or indirect) regulator of ER gene expression than is the wild type ER. This notion is consistent with the results we previously reported, which show that seven of the eight B-variant tumors had relatively low ER mRNA levels (wild type plus variant). (See ref. 1, Fig. 6.) A direct test of the variant's activity as a transactivator could be made by constructing an expression vector containing the ER B-variant sequence and comparing ER variant and wild type activity in transfected cells.

The observed heterozygosity frequency of 12.2% implies a homozygosity frequency of 0.4% or 1 in 250 individuals. Therefore, the fact that we have not encountered a homozygote among the 483 samples tested so far is interesting. But to state with 95% confidence that the ER B-variant homozygote is not viable, at least 960 individuals must be tested. Given the relative ease of detecting the B-variant by the PCR/ASO assay, this information should be feasible to obtain.

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